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Biomolecular Surface Engineering of Pancreatic Islets with Thrombomodulin

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Abstract

Islet transplantation has emerged as a promising treatment for Type 1 diabetes, but its clinical impact remains limited by early islet destruction mediated by prothrombotic and innate inflammatory responses elicited upon transplantation. Thrombomodulin (TM) acts as an important regulator of thrombosis and inflammation through its capacity to channel the catalytic activity of thrombin towards generation of activated protein C (APC), a potent anti-coagulant and anti-inflammatory agent. We describe herein a novel biomolecular strategy for re-engineering the surface of pancreatic islets with TM. A biosynthetic approach was employed to generate recombinant human TM (rTM) bearing a C-terminal azide group, which facilitated site-specific biotinylation of rTM through Staudinger ligation. Murine pancreatic islets were covalently biotinylated through targeting of cell surface amines and aldehydes, and both islet viability and the surface density of streptavidin were maximized through optimization of biotinylation conditions. rTM was immobilized on islet surfaces through streptavidin-biotin interactions, resulting in a nearly three-fold increase in the catalytic capacity of islets to generate APC.

Keywords

Islet Transplantation; Thrombomodulin; Cell Surface Engineering; Staudinger Ligation

1. Introduction

Islet transplantation has emerged as a promising cell-based therapy for the treatment of Type 1 diabetes [1–3]. However, the clinical efficacy of islet transplantation remains limited, in part, by the destruction of islets in the immediate post-transplant period [4–6]. Consequently, it is often necessary to transplant islets from 2 to 4 donor organs to reverse diabetes in a single

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patient [2,3,7,8], further burdening a limited donor islet source [9], increasing health care costs [10], and the incidence of procedural complications. Results from clinical islet transplantation and experimental animal models provide compelling evidence that early islet destruction is largely mediated by innate inflammatory responses characterized by activation of endothelial cells (ECs) [11], intense infiltration of leukocytes into and around islets [11–13], and elevated levels of proinflammatory mediators [11,14,15] that adversely affect β-cell viability and function [16,17].

Unlike conventional biomaterials, which are largely passive bystanders of inflammatory/foreign body responses and subsequent device failure [18], islets actively contribute to their own destruction via expression of mediators that initiate inflammatory and procoagulant pathways. This response is generated by an instantaneous blood-mediated inflammatory reaction (IBMIR) triggered upon infusion of islets into the portal vein of the liver, the clinically preferred site for islet transplantation [19–21]. Korsgren and colleagues have demonstrated that islets express tissue factor (TF), which initiates the extrinsic blood coagulation pathway by complexing with factor VIIa, catalyzing the conversion of factor X to its active form, fXa, accelerating the conversion of prothrombin to thrombin. Indeed, islets incubated in non-anticoagulated blood induce a significant thrombotic response, as evidence by fibrin clots surrounding islets and increased levels of thrombin-antithrombin complex (TAT), prothrombin fragments 1 and 2, fXIa-antithrombin complex, and β-thromboglobulin [19,20]. Additionally, despite the presence of heparin in the infusate, serum levels of prothrombotic markers (TAT, fVIIa-antithrombin, and D-dimer) were significantly elevated 15 minutes to 24 hours after islet transplantation [21]. Thrombin acts as an important mediator of cellular responses during inflammation, triggering the expression of EC adhesion molecules [22] and production of proinflammatory cytokines [23]. Furthermore, thrombin acts as a chemoattractant [24] and activates platelets, which release alpha-granule chemokines and express P-selectin, thereby attracting neutrophils and monocytes and promoting their arrest and activation [23,25,26]. Accordingly, EC activation, neutrophil and macrophage infiltration, and increased production of cytokines and inflammatory mediators are observed 6 to 12 hours after transplantation in animal models of islet transplantation, resulting in significant islet apoptosis within 24 hours [11,12]. Hence, islet-initiated thrombin generation contributes significantly to the initiation and/or elaboration of inflammatory responses implicated in early islet destruction.

Under normal physiological conditions, endothelial cells lining the extensive microvasculature of pancreatic islets actively regulate coagulation [27]. During islet isolation and culture, however, this barrier is disrupted [27,28], exposing procoagulant and inflammatory mediators while simultaneously stripping away EC-derived regulators of thrombosis including heparin, CD39, and thrombomodulin (TM). TM, a 60 kD type I transmembrane protein, is the most important physiological regulator of coagulation in the microcirculation and acts as an important link between coagulation and inflammation [29]. TM forms a 1:1 molar complex with thrombin, thereby sequestering it from participating in thrombotic and inflammatory processes, while simultaneously redirecting its catalytic activity towards generation of activated protein C (APC) [29]. APC directly inhibits generation of factors VIIIa and Va, further inhibiting generation of thrombin [29,30]. APC also possesses potent, coagulation-independent anti-inflammatory activity [29], inhibiting macrophage production of proinflammatory cytokines [31–33], endothelial cell expression of E-selectin and ICAM-1 [34], and neutrophil binding to selectins [35].

Given these observations, we have postulated that administration of TM represents a rational strategy for inhibiting thrombotic and inflammatory processes that underlie early islet destruction. Indeed, we have recently reported that intravenous administration of a liposomal formulation of TM (lipo-TM) enhanced islet engraftment in a murine model of intraportal islet transplantation [36]. While the half-life of lipo-TM was found to be significantly longer than

that of systemically administered APC [37], repetitive dosing would likely be necessary to sustain TM activity. By contrast, TM immobilized on surfaces facilitates sustained generation of APC in the presence of thrombin with attenuation of thrombotic and inflammatory responses initiated by islet-derived tissue factor (Scheme 1) [38,39]. Towards this end, we describe herein a novel strategy for immobilizing recombinant human TM (rTM) to the islet surface in a site-specific manner using well-established biotin-avidin interactions. Moreover, unique covalent islet surface modification techniques were employed, which have broad implications for chemically remodeling cell and tissue surfaces. Finally, through optimization of islet surface biotinylation and subsequent immobilization of biotinylated rTM, the catalytic capacity of islets to generate APC was increased nearly three-fold.

2. Materials and Methods

2.1. Animals

Male C57BL/6J (B6) and B10.BR-H2k H2-T18a/SgSnJ (B10) mice (8 weeks old, Jackson Laboratory Bar Harbor, ME) were used as islet donors. All animal studies followed local Institutional Animal Care and Use Committee guidelines at Emory University.

2.2. Pancreatic islet isolation

Pancreatic islet isolations were performed, as previously described [40]. B10 or B6 mice pancreata were removed after distension with collagenase P (1 mg/ml, Roche, Indianapolis, IN) through the common bile duct. Following digestion, islets were purified by a Ficoll-Histopaque discontinuous gradient (Ficoll: 1.108, 1.096, and 1.037; Mediatech Inc., Manassas, VA). Isolated islets were cultured for 48 to 72 hours at 37°C in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml; Mediatech Inc.), and media was changed daily. Purity was determined to be >90% after 24 hours of culture and islets were handpicked from the preparation for all investigations to further increase islet purity.

2.3. Measurement of thrombomodulin activity

The cofactor activity of thrombomodulin (TM) on islets was determined by measuring the production of activated protein C (APC) in the presence of protein C, thrombin, and calcium. Groups of 40 to 50 islets isolated from B6 mice were handpicked under a dissecting microscope, placed into wells of a 96 well plate with 75 μL of 20mM Tris-HCI (pH 7.4) containing 1 μM human protein C (Calbiochem, San Diego, CA), 1 nM alpha-thrombin (Haematologic Technologies, Essex Junction, VT), 5 mM $CaCl_2$, 100 mM $NaCl_2$, and 0.1% (wt) BSA. After 1 hr incubation at 37°C, production of APC was quenched for 5 minutes by the addition of 2 IU/mL antithrombin III (American Diagnostica, Stamford, CT). Thirty microliter samples were collected and APC was detected by the addition of 0.5 mM Spectrozyme PCa (American Diagnostica). Absorbance measurements at 405 nm were recorded every 30 seconds for 40 minutes to determine the rate of chromogenic substrate conversion by APC. APC concentration was determined using a standard curve relating rates of chromogenic substrate conversion to known concentrations of APC (American Diagnostica) and normalized by islet number.

2.4. Expression and purification of recombinant human azido-thrombomodulin

A DNA fragment encoding for the EGF (4–6) domains of human TM was obtained by polymerase chain reaction (PCR) using the primers 5'-GTGGAACCGGTTGACCCGTGCT-3' and 5'-TTATTACATGCCACCGTCCACCTTGCC-3'. Site-directed mutagenesis was used to mutate the single internal methionine residue to leucine at position 388. PCR was used to create a C-terminus GlyGlyMet coding region. The final construct was inserted into the pFLAG ATS

expression system (Sigma, St. Louis, MO) at HindIII. rTM-N_3 was expressed in the *E. coli* methionine auxotroph B834 in minimal media supplemented with homoazidoalanine, synthesized as described previously [41]. Control rTM-methione was expressed using the same E. coli methionine auxotroph in Luria Bertani (LB) media. rTM was purified with immunoaffinity chromatography using anti-FLAG affinity gel (Sigma Aldrich).

2.5. Synthesis of biotin-PEG-triphenylphosphine

A triphenylphosphine-poly(ethylene glycol)-biotin conjugate was synthesized by reaction of a heterobifunctional biotin-$PEG_{3.4kD}$-amine linker (CreativePEGWorks, Winston Salem, NC) with a pentafluorophenyl (PFP) active ester of triphenylphosphine, synthesized as described previously [42,43]. To a stirred solution of biotin-$PEG_{3.4kD}$-amine (100 mg, 0.029 mmol) in dichloromethane (DCM; 2 mL) was added the PFP-ester of triphenylphosphine (31.17 mg, 0.058 mmol, 2 equiv) and Et_3N (8.08 μl, 2 equiv.), and the resultant mixture stirred at room temperature for 12 to 16 hr, upon which time volatiles were evaporated by vacuum. The residue was dissolved in the minimum amount of cold DCM and the product was precipitated by cold ether. The pure compound was collected by filtration and dried by vacuum. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.45 (m, 2H), 1.6–1.8 (m, 4H), 2.2 (t, *J* = 7.6 Hz, 2H), 2.8 (d, *J* = 12.8 Hz, 1H), 2.9 (dd, *J* = 4.8, 12.8 Hz, 1H), 3.2 (m, 1H), 3.3–3.9 (m, PEG), 3.7 (s, 1H), 4.3 (m, 1H), 4.5 (m, 1H), 6.7 (m, 2H), 7.2–7.4 (m, 11H), 7.8 (dd, *J* = 1.6, 8.4 Hz, 1H), 8.1 (dd, *J* = 4, 8.4 Hz, 1H).

2.6. Site-specific biotinylation of recombinant TM

Purified azido-functionalized rTM was mixed with biotin-PEG-triarylphosphine (1:500 molar ratio) in PBS (pH 7.4), and the reaction mixture incubated at 37°C for 48 hr (Scheme 2). Conjugation was monitored using SDS-PAGE (10%) and Commassie total protein stain. Excess linker was removed by Amicon ultrafiltration using a 10,000 Da MWCO filter (Millipore, Billerica, MA), with additional purification achieved through anti-FLAG chromatography to capture the rTM. The final desired rTM-biotin product was obtained after monomeric avidin chromatography (Pierce Biotechnology). Total protein was quantified with the Bradford protein assay (Bio-Rad, Hercules, CA). Biotinylation was confirmed using the FluoReporter Biotin Quantitation Assay Kit (Molecular Probes, Eugene, OR).

2.7. Biotinylation of pancreatic islets

N-hydroxysuccinimide (NHS) esters and hydrazide-functionalized reagents were used to biotinylate cell surface amines and aldehydes, respectively. Prior to biotinylation, islets (<1000) were placed into 12 mm cell culture inserts with 12 μm pores (Millicell-PCF; Millipore), and washed six times by adding 700 μL of Dulbecco's phosphate buffered saline containing calcium and magnesium (DPBS) to the insert, followed by gentle repeated tapping of the insert on a polystyrene surface to facilitate drainage of the wash solution through pores while retaining islets. NHS-$PEG_{3.4kD}$-biotin (Nektar Therapeutics, Huntsville, AL) or sulfosuccinimidyl-6-(biotinamido) hexanoate (sNHS-LC-biotin; Pierce Biotechnology) were used to biotinylate islet surface amine groups. Compounds were dissolved at the desired concentration in DPBS supplemented with 11 mM glucose (DPBSG) and added to islets within 10 seconds of dissolution to minimize ester hydrolysis. Reactions were performed for one hour at room temperature and islets rinsed six times, as described above, to remove unreacted biotin.

Islet surface aldehyde groups were generated through periodate oxidation of *cis*-glycol groups. Islets were incubated in 1 mM sodium metaperiodate ($NaIO_4$; Pierce Biotechnology) in DPBS protected from light for 15 minutes. Islets were then rinsed six times with DPBS and subsequently incubated in biotinamidohexanoic acid hydrazide (hydrazide-LC-biotin; Pierce Biotechnology) at the desired concentration and reaction time. Islets were then rinsed an additional six times to remove unreacted reagent.

2.8. Assessment of islet viability

Islet viability was assessed as previously described [44]. Briefly, a representative population of islets were incubated in DPBS containing 4 μM calcein AM and 8 μM ethidium homodimer-1 (Molecular Probes, Eugene, OR) for one hour, and individual islets were imaged at approximately their equatorial plane with two-channel confocal microscopy (Zeiss LSM 510 META; Carl Zeiss, Inc., Thornwood, NY) using a 20× objective. Confocal micrographs were converted to binary images comprised of red and green pixels and analyzed using MATLAB® (The MathWorks, Natick, MA) to quantify the number of pixels corresponding to fluorescent emission from live (green) and dead (red) cells. Viability is calculated as the percentage of total fluorescent pixels that are green and an average viability is determined by performing this analysis on 35–50 images of individual islets.

2.9. Quantification of immobilized streptavidin

Following biotinylation, islets were incubated in a mixture of HRP-labeled streptavidin (HRP-SA; Zymed Laboratories, Inc., San Francisco, CA) and streptavidin (Pierce Biotechnology) at 0.1 mg/mL (1:50 w/w) in DPBSG for 30 minutes. After washing islets, groups of 30–50 islets were placed into wells of a 96 well plate. The microplate was briefly centrifuged to settle islets, supernatant was removed, and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (1-Step™ Ultra TMB-ELISA, Pierce Biotechnology) was added to each well. Microwell plates containing islets and TMB were placed on a plate shaker (800 min^{-1}, MS1 Minishaker, IKA, Wilmington, NC) at room temperature for 20 min, upon which time 50 μL 2M H_2SO_4 was added to quench the reaction. Microwell plates were briefly centrifuged to settle islets, 100 μL of solution was transferred to a fresh well, and absorbance was recorded at 450 nm using a microplate reader. The amount of streptavidin immobilized on islets was quantified using a standard curve relating absorbance at 450 nm to known concentrations of soluble SA-HRP.

2.10. Immobilization of rTM on mouse islets

Following biotinylation, islets were incubated with 0.1 mg/mL streptavidin (Pierce Biotechnology) in DPBSG for 30 minutes. Islets were washed with DPBSG six times, as described above, to remove free streptavidin. Islets were then incubated with the rTM-$PEG_{3.4kD}$-biotin (rTM-biotin) conjugate (3.5 μM in DPBSG) for one hour at room temperature. Islets were then washed eight times to remove free rTM-biotin prior to measuring TM activity.

2.11. Statistics

Tests for statistical significance between the means of two groups were conducted with the Student's t-test (two-tailed, homoscedastic). Tests between three or more groups were conducted with the one-way ANOVA followed by the Tukey HSD test.

3. Results and Discussion

3.1. Site-specific biotinylation of recombinant human thrombomodulin

Cell surface immobilization of exogenous TM on pancreatic islets provides a rational approach to increasing local concentrations of APC at the site of transplantation. Towards this objective, we sought to generate a biotinylated TM that could be readily immobilized on islet surfaces via biotin-(strept)avidin interactions. Though TM activity is maximized when inserted into a phospholipid bilayer [45], it is well established that the extracellular EGF-like domains 4–6 of human TM possess full cofactor activity [46]. Hence, a biosynthetic approach was used to generate rTM containing these domains, as well as a non-canonical, C-terminal azido-methionine analog (rTM-N_3) [47,48]. Site-specific biotinylation was achieved through chemoselective Staudinger ligation between triphenylphosphine-derivatized poly(ethylene glycol)-biotin and the C-terminal azide of rTM-N_3. While biotinylation of proteins is routinely

performed, generally through targeting of amino groups, the exquisite orthogonality of the Staudinger ligation [49] provides a strategy for biotinylation in a site-specific manner, thereby eliminating loss of protein activity associated with covalent modification of amino acids within the active site. Indeed, we have previously demonstrated that TM activity is not compromised upon site-specific conjugation of poly(ethylene glycol) [48]. Moreover, through incorporating biotin at the C-terminus using a 3.4kD PEG spacer, the molecular accessibility of thrombin to immobilized rTM may be increased. Thus, rTM may be linked to streptavidin in a manner that more closely mimics the presentation of TM as it appears on the cell surface [29], which may consequently maximize activity in the immobilized state.

Upon reaction, SDS-PAGE of the crude mixture demonstrated the presence of two species separated by approximately 4 kD, corresponding to the desired conjugate (TM-PEG-biotin) and unreacted rTM-N_3 (Fig. 1A). By contrast, only a single band was observed when rTM bearing a C-terminal methionine was used, demonstrating the chemical specificity of the conjugation. Densitometry indicated that approximately 50% of rTM-N_3 had been conjugated to the biotin-PEG linker. Conjugation of PEG-biotin to only 50% of rTM may be attributed to several factors. First, despite the high specificity of the Staudinger ligation, competing oxidation of phosphine in aqueous solvent, coupled with steric hindrance associated with coupling between two relatively large species (~4kD and ~20kD) each with a single reactive site, serve to reduce the efficiency of this reaction. Second, the incorporation of homoazidoalanine into the amino acid sequence of rTM is not completely efficient, yielding a fraction of rTM that does not contain azide groups, and, hence, is recalcitrant to biotinylation through Staudinger ligation. However, upon subsequent purification with centrifugal dialysis and monomeric avidin chromatography, immunoblotting against thrombomodulin demonstrated the presence of a single species of molecular weight corresponding to the desired rTM-PEG-biotin conjugate (Fig. 1B). Biotinylation was confirmed with Western blotting using HRP-labeled streptavidin (Fig. 1C).

3.2.Maximizing streptavidin binding through optimization of cell surface biotinylation

Covalent biotinylation of pancreatic islets has been employed previously to tether macromolecules on the islet surface [50,51]. However, little work has been done to optimize reaction schemes or conditions with the objective of maximizing the surface density of biotin moieties, or subsequently immobilized (strept)avidin, while maintaining high islet viability. To date, covalent modification of islet surfaces has been accomplished nearly exclusively through amine-reactive chemistries, most commonly NHS-esters [50–53], though the dependence of conjugation efficiency on important reaction conditions, most notably concentration, are rarely investigated or reported. Additionally, cell-surface carbohydrates, in particular sialic acid residues, may be covalently modified through mild periodate oxidation of *cis*-glycol groups and subsequent hydrazone linkage between resultant aldehydes and hydrazine-activated molecules [54–56]. While this approach has been used to modify a variety of cell types, its utility for chemically re-engineering the surface of pancreatic islets has not been explored. Therefore, in an effort to maximize the amount of rTM-biotin that may be immobilized on islets, the capacity of both NHS-ester and aldehyde-hydrazide biotinylation strategies to facilitate immobilization of streptavidin was investigated (Scheme 3).

The biotinylation conditions investigated are summarized in Table 1. To explore the effect of a poly(ethylene gycol) (PEG) spacer arm between the covalent linkage and biotin moiety, islets were reacted with either NHS-$PEG_{3.4kD}$-biotin or sulfosuccinimidyl-6-(biotinamido) hexanoate (sNHS-LC-biotin) at 4 mM for 1 hour, and the amount of immobilized streptavidin (SA) compared. Use of NHS-$PEG_{3.4kD}$-biotin yielded significantly less SA than sNHS-LC-biotin ($p<0.05$, Fig. 2A), potentially due to generation of a steric barrier with increasing density of PEG chains on the islet surface [57]. Based on these findings, sNHS-LC-biotin was used to

investigate the effect of concentration on conjugation efficiency. Increasing sNHS-LC-biotin concentration to 20 mM did not have a significant effect on the amount of surface-bound SA (Fig. 2A), suggesting saturation of SA surface density through this approach. Increasing reaction time beyond 1 hour was not explored, as hydrolysis of NHS-esters occurs rapidly and is reported to be nearly complete within an hour.

Though less commonly employed, coupling between cell surface aldehydes and hydrazide-derivatized molecules offers an alternative to amine-reactive chemistries, and, therefore, was investigated as a means to biotinylate islets. Islets were treated with 1 mM $NaIO_4$ for 15 minutes to generate cell surface aldehyde groups [56], and subsequently reacted with 4 mM hydrazide-LC-biotin for 1 and 3 hours. No statistical difference ($p > 0.05$) in immobilized SA was detected between 1 and 3 hour incubation times (Fig. 2A), suggesting that hydrazone bond formation between hydrazide-LC-biotin and cell surface aldehydes approaches equilibrium after an hour. Increasing the concentration of hydrazide-LC-biotin to 20 mM resulted in a significant increase in SA binding ($p < 0.05$), yielding levels statistically comparable ($p > 0.05$) to optimized NHS-LC-biotin coupling. Exploration of higher concentrations was not possible due to the solubility limit of hydrazide-LC-biotin.

It was next postulated that biotin surface density might be further increased through combination of amine- and aldehyde-reactive coupling strategies. To investigate this possibility, islets were serially biotinylated using conditions optimized for each strategy. Islets were first treated with 1 mM $NaIO_4$ for 15 minutes, reacted with 20 mM hydrazide-LC-biotin for 1 hour, and finally reacted with 4 mM NHS-LC-biotin for 1 hour. This combination approach yielded a significant increase ($p<0.05$) in SA density of approximately 50% relative to either treatment alone (Fig. 2A). Interestingly, the relative contributions from each conjugation strategy were not additive, suggesting molecular crowding or surface saturation. Confocal microscopy of islets incubated with Cy3-labeled streptavidin after combination biotinylation (Fig. 2B) confirmed that streptavidin was localized on cell surfaces. These results demonstrate that the surface density of streptavidin, and, consequently, the surface density of biotinylated macromolecules, may be increased through chemically targeting multiple reactive groups on the cell surface. Significantly, to our knowledge this is the first report describing chemical targeting of *both* amine and aldehyde groups on the surface of cells or tissue.

Given the role of cell surface proteins and carbohydrates in diverse biochemical processes critical to cell survival, covalent modification of the islet surface may have detrimental impacts on islet viability. While previous reports demonstrate that islet viability and function are maintained upon biotinylation and subsequent immobilization of (strept)avidin [50,51], suboptimal reaction conditions were used and aldehyde-hydrazide coupling was not explored. Therefore, the viability of islets biotinylated through targeting in combination surface amines and aldehydes and subsequently incubated with streptavidin was assessed. Combination treatment had no discernable impact on islet viability relative to non-treated controls, both immediately after treatment (treated: $97.2 \pm 2.0\%$, n=48 islets vs. untreated: $98.4 \pm 2.0\%$, n=42; $p > 0.01$), as well as after 24 additional hours in culture post-treatment (treated: $98.3 \pm 1.6\%$, n=38 vs. untreated: $98.3 \pm 2.0\%$, n=40; $p > 0.05$) (Fig. 3). While this provides evidence of high islet viability in the period wherein modified islets would likely be transplanted in practice (i.e., 0–24 hrs post-treatment), further elucidation of the effect of surface modification on islet function and long-term viability should be assessed in a murine model of intraportal islet transplantation; this is an area of ongoing investigation.

3.3. Immobilization of rTM on islets increases rates of protein C activation

Maximizing the amount of streptavidin on the islet surface is anticipated to facilitate immobilization of a high density of rTM-biotin, with an attendant increase in the ability of islets to activate protein C. Islets were biotinylated via combination treatment, incubated with

0.1 mg/mL streptavidin for 30 minutes followed by incubation with rTM-biotin at 3.5 μM for one hour. Upon extensive rinsing of treated islets to remove unbound rTM-biotin, APC generation was measured and compared to untreated islets, islets treated with non-modified rTM (i.e., without biotin), and islets treated only with biotinylation reagents and streptavidin as controls (Fig. 4). Treatment of islets with rTM-biotin resulted in an approximately three-fold increase in APC production relative to untreated controls, which were capable of activating protein C as a result of endogenous expression of TM [58]. No significant difference in APC generation relative to untreated controls was observed after biotinylation and subsequent immobilization of streptavidin or treatment with non-biotinylated rTM, indicating that the observed increase in APC production is not an artifact of increased endogenous TM expression or non-specific binding of rTM to the islet surface, but rather a consequence of rTM-biotin incorporation. Hence, cell surface immobilization of rTM significantly increases the capacity of islets to activate protein C.

We have previously reported an alternative approach to tethering rTM to islet surfaces through a two-step process in which a heterobifunctional N-hydroxysuccinimide-PEG-triphenylphosphine was covalently linked to cell surface amines and subsequently used to directly immobilize rTM-N_3 through Staudinger ligation [59]. While this purely covalent approach eliminated the use of streptavidin, a possible immunogen, we postulated that it may be limited, in part, by inefficient conjugation between cell surface- phosphine moieties and azide groups on rTM. Moreover, the approach presented herein enabled use of both NHS and hydrazide coupling strategies, thereby increasing the surface density of available anchoring sites for rTM-biotin. Indeed, while we had previously reported an approximately 7-fold increase in APC generation relative to untreated controls, the *net* increase in APC generation attributed to immobilized rTM-biotin is 4-fold higher in the current approach.

Whether the observed increase in APC generation will be sufficient to improve the outcome of intraportal islet transplantation is an area of ongoing investigation. It should be emphasized that the observed three-fold increase may be significantly higher upon islet transplantation as endogenous TM expression is likely to decrease in response to islet-mediated inflammatory responses. Moreover, several investigators have observed that inflammatory stimuli, similar to those generated upon intraportal islet transplantation, decrease thrombomodulin expression in hepatic sinusoidal endothelial cells [60,61], thereby, further decreasing APC production within the transplant microenvironment. Indeed, Ishii *et al*. have reported a 50% reduction in TM activity by endothelial cells upon exposure to the inflammatory cytokine TNF-α [62]; a three-fold increase in TM activity would have completely mitigated this effect. Moreover, Liaw *et al*. [63] have reported a significant, but modest, increase in baseline APC levels in survivors of severe sepsis relative to nonsurvivors (5.3 ng/mL vs. 3.7 ng/mL). While different pathologically than inflammatory events elicited by islet transplantation, this report exemplifies the potential significance of even modest increases in APC generation in affecting clinical outcomes. As such, conjugation of TM to islets provides a strategy for physically targeting TM to the site of islet transplantation, potentially restoring local TM activity to basal levels. Interestingly, it has recently been reported that surface heparinization of intraportal islet grafts reduced TAT production and early islet damage in an allogenic porcine model [50]. While a direct comparison with soluble heparin was not made, in light of the inefficacy of systemically administered heparin during clinical islet transplantation [2,20,21], these findings potentially illustrate the increased therapeutic benefit achieved through local delivery of anticoagulants to the portal bed. Given the increased capacity of TM to inhibit thrombin generation relative to heparin, similar or more substantial effects might be reasonably anticipated.

4. Conclusions

Though intrahepatic infusion of islets remains the clinical standard for islet transplantation, direct contact between islet-derived tissue factor and blood initiates thrombosis and inflammation in the immediate post-transplant period with deleterious consequences to islet survival and function. We have presented a strategy for conferring anticoagulant potential to islets through immobilization of rTM on the islet surface. Through site-specific, C-terminal biotinylation of rTM and optimization of cell surface biotinylation strategies that target both amine and aldehyde groups, integration of rTM resulted in an approximately three-fold increase in the catalytic capacity of islets to activate protein C. Conjugation of TM to islets represents a facile strategy for increasing APC generation at the site of transplantation, and the localized delivery of anticoagulants offers the potential to increase rates of islet survival and function with attendant improvements in clinical outcomes.

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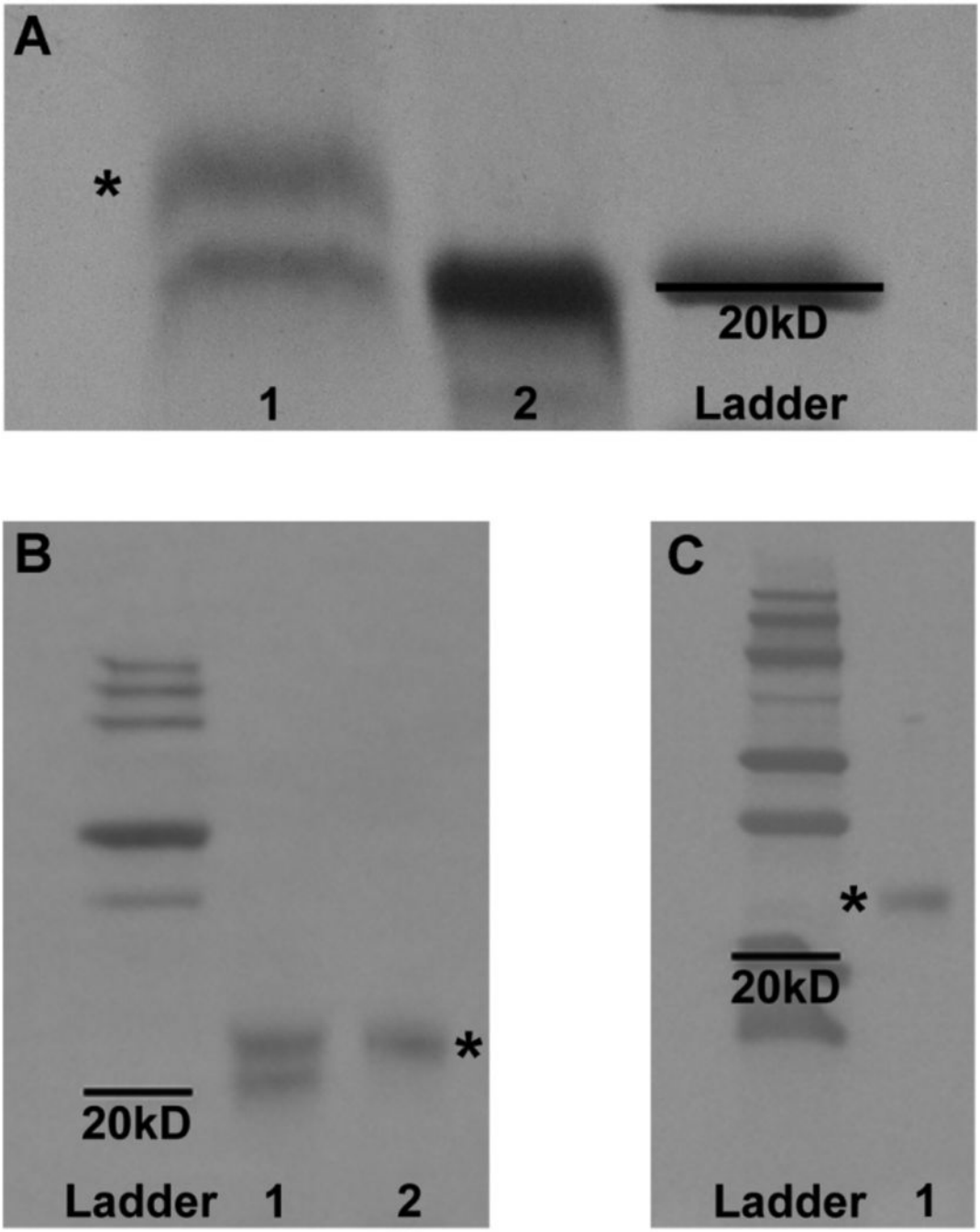


Figure 1. Site-specific biotinylation of recombinant human thrombomodulin

(A) Upon reaction between rTM-N_3 and triphenylphosphine-$PEG_{3.4kD}$-biotin, SDS PAGE reveals the presence of two species separated by approximately 4 kD (Lane 1), corresponding to the desired biotinylated conjugate (*) and unreacted rTM-N_3. A molecular weight shift was not observed in a parallel control reaction using rTM engineered without an azido group (Lane 2), demonstrating the specificity of the Staudinger ligation. **(B)** Western blot against human TM after initial conjugation (Lane 1) and subsequent purification (Lane 2). After purification via centrifugal dialysis and monomeric avidin chromatography, a single species corresponding to the expected molecular weight of the desired biotin-PEG-TM conjugate is observed (*).

(C) Western blot against biotin using HRP-labeled streptavidin confirms biotinylation of the construct (*; Lane 1).

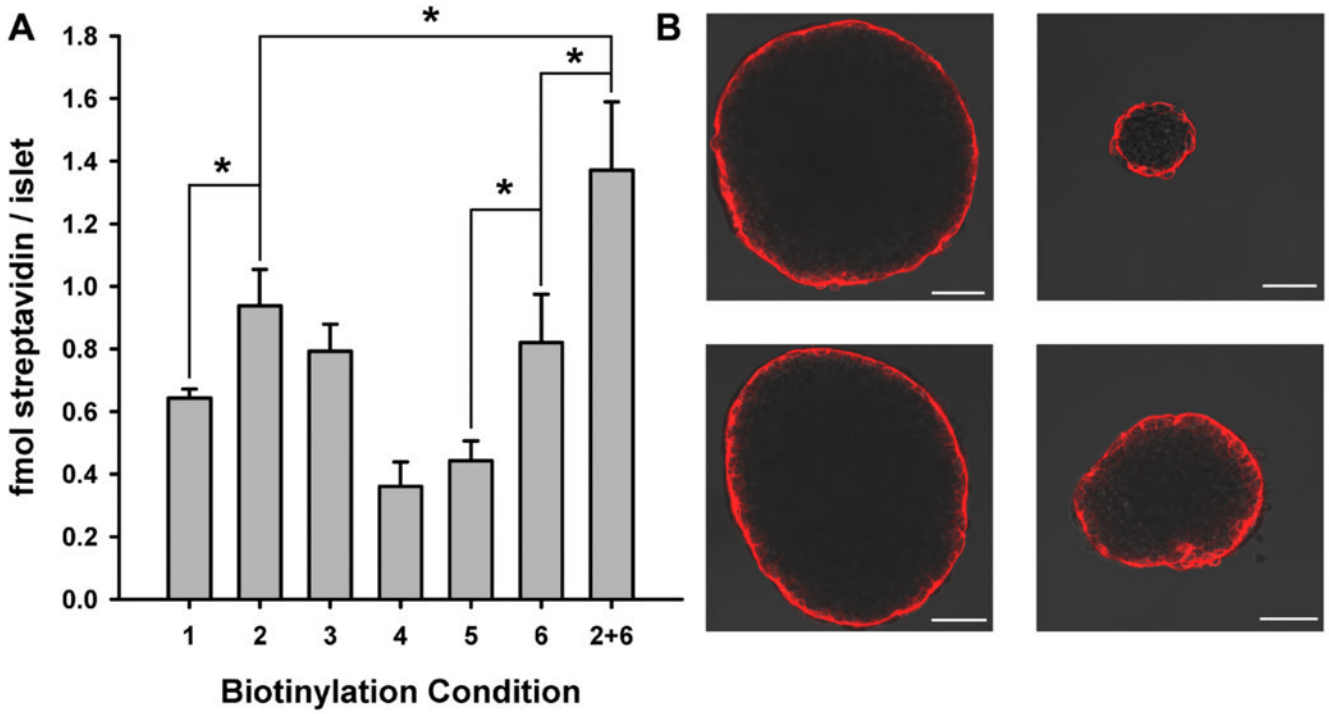


Figure 2. Islet surface density of streptavidin may be maximized through optimization of biotinylation reactions targeting cell surface amines and aldehydes

(A) Amount of streptavidin immobilized on the islet surface (mean ± SD) after surface biotinylation using conditions summarized in Table 1 (*$p > 0.05$). **(B)** Representative confocal micrographs of islets incubated with Cy3-labeled streptavidin (red) after combination biotinylation (i.e., condition 2+6) overlaid on bright field micrograph (scales bar 50 μm).

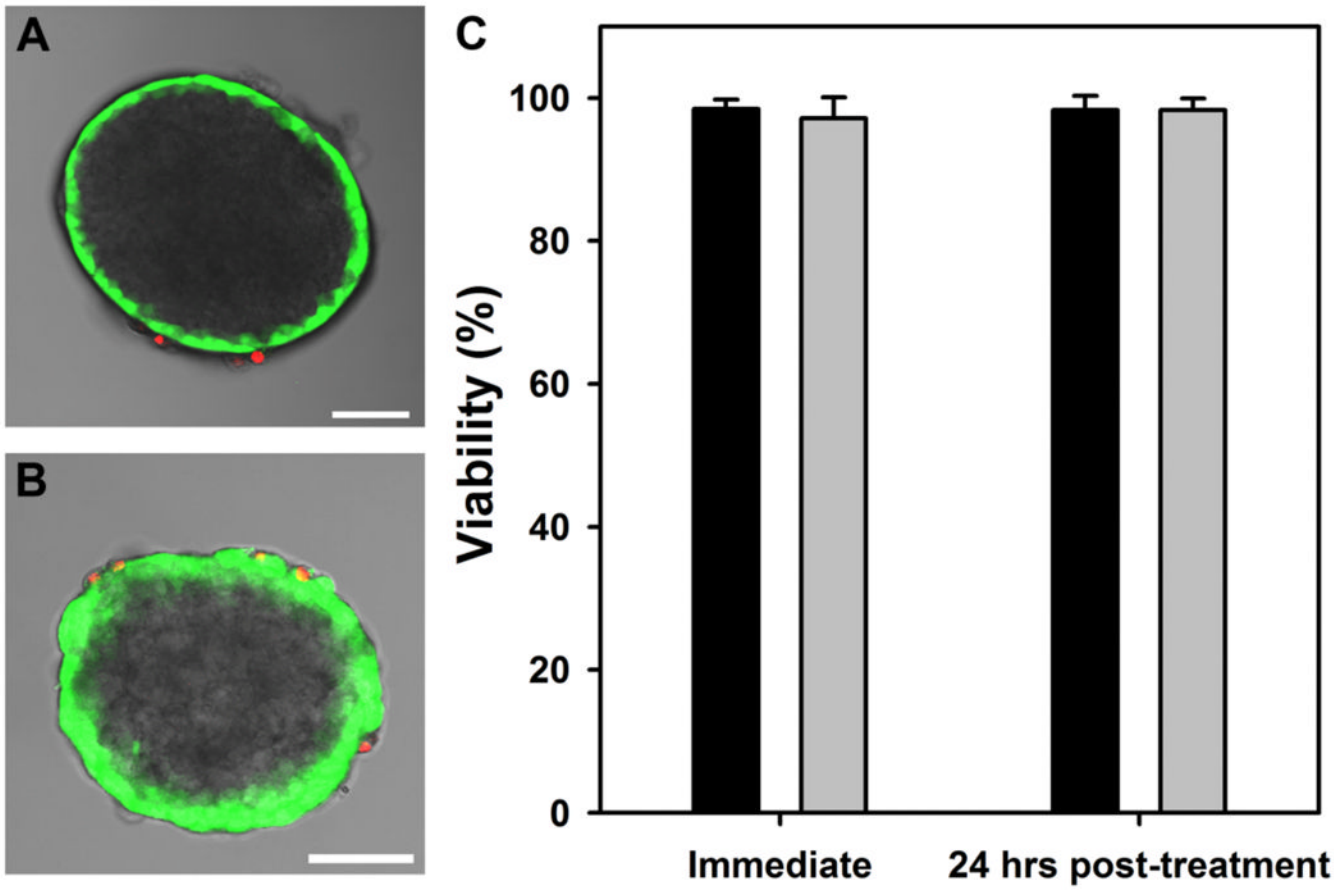


Figure 3. Islet viability before and after biotinylation of surface amines and aldehydes

Representative confocal micrographs of untreated islets **(A)** and biotinylated islets **(B)** incubated with calcein AM (green, live) and ethidium homodimer-1 (red, dead) overlaid on bright field micrograph (scale bar 50 μm). **(C)** Islet viability (mean ± standard deviation) of untreated islets (black bars) and islets biotinylated via combination treatment (grey bars) immediately after treatment and 24 hours later.

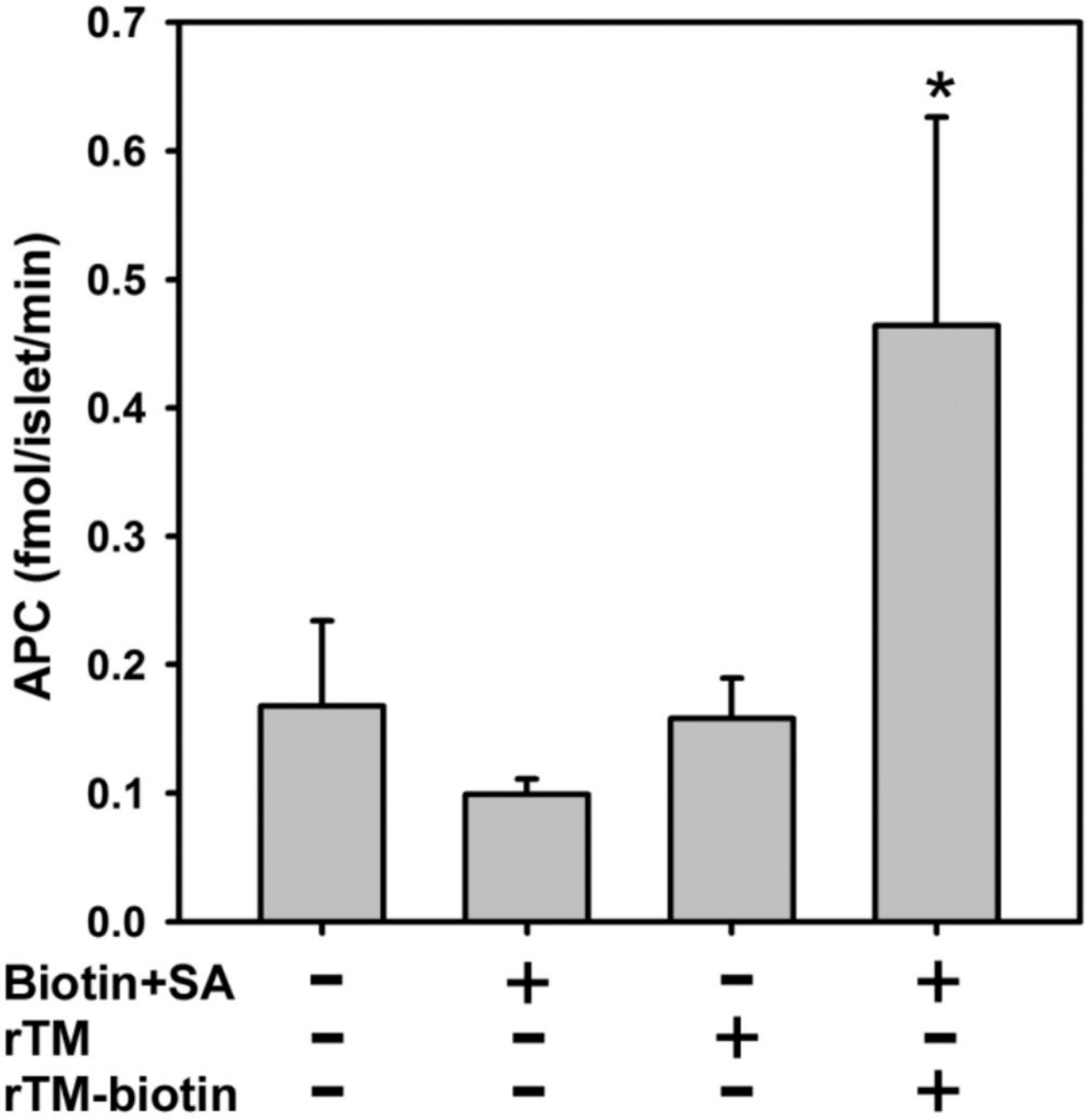
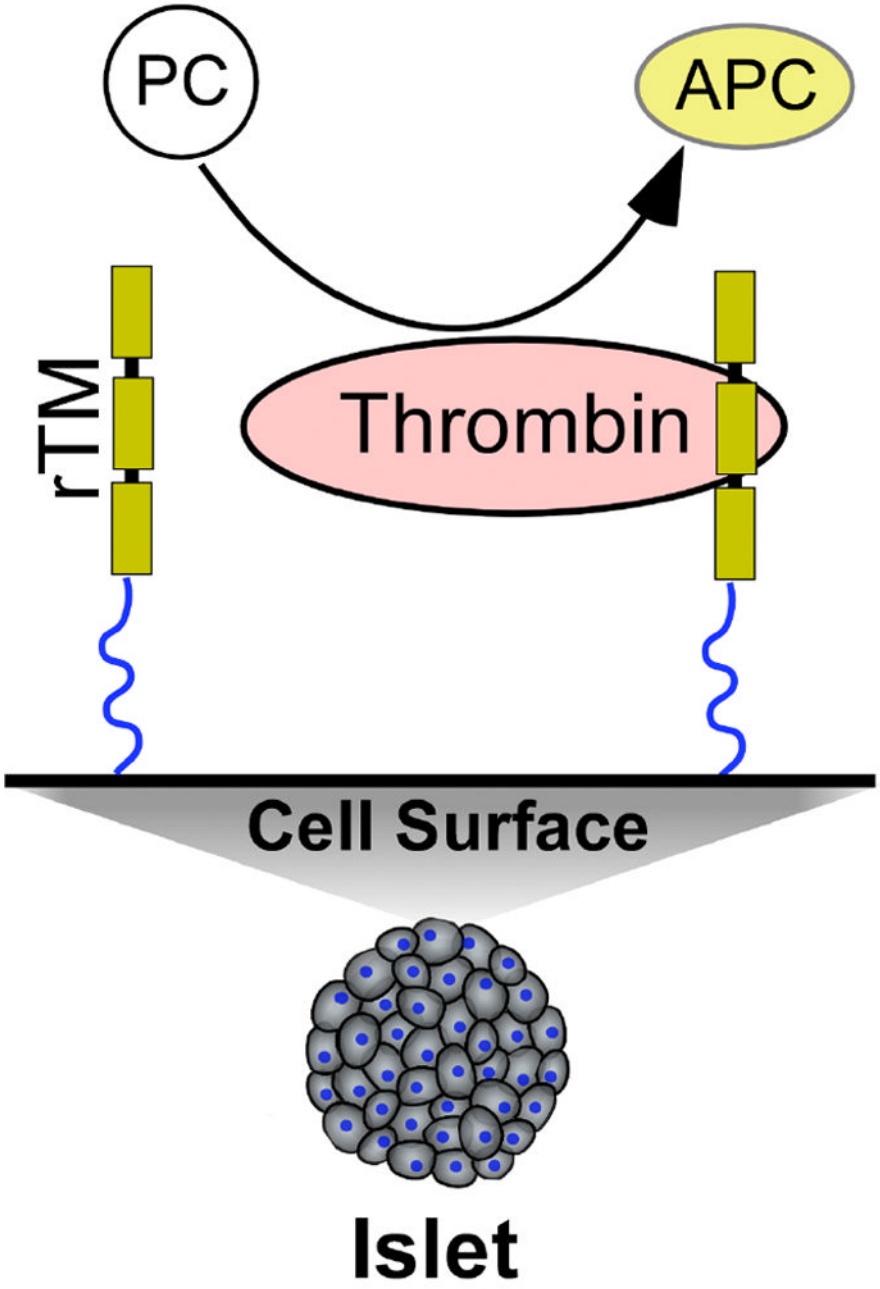
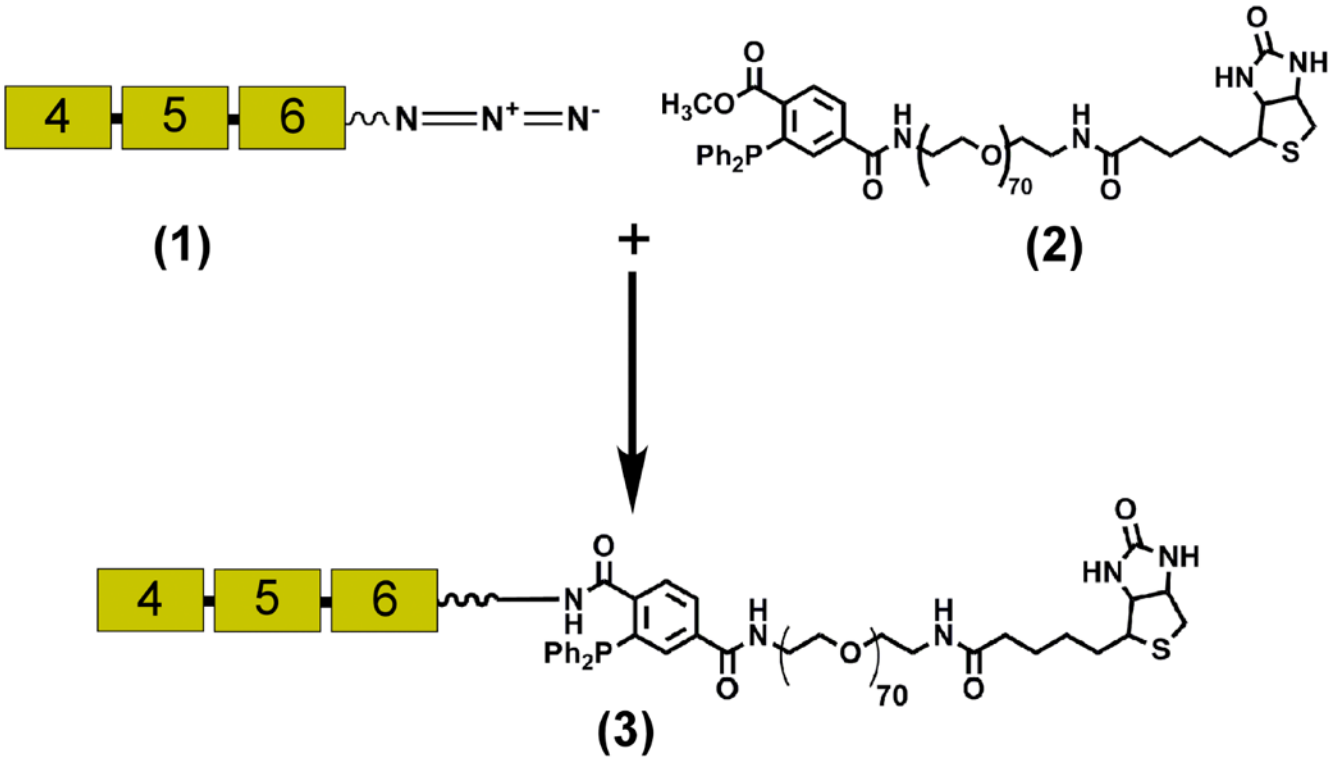


Figure 4. Immobilization of rTM on the islet surface via streptavidin-biotin interactions increases rates of activated protein C (APC) generation

Upon combination biotinylation and subsequent incubation with streptavidin (Biotin + SA) islets were incubated with rTM-biotin at 3.5 μM for 1 hour, resulting in an approximately three-fold increase in the rate of APC generation relative to untreated controls (*$p<0.05$). Immobilization of streptavidin alone or incubation of untreated islets with rTM without biotin was found to have no effect on rates of APC generation ($p>0.05$).

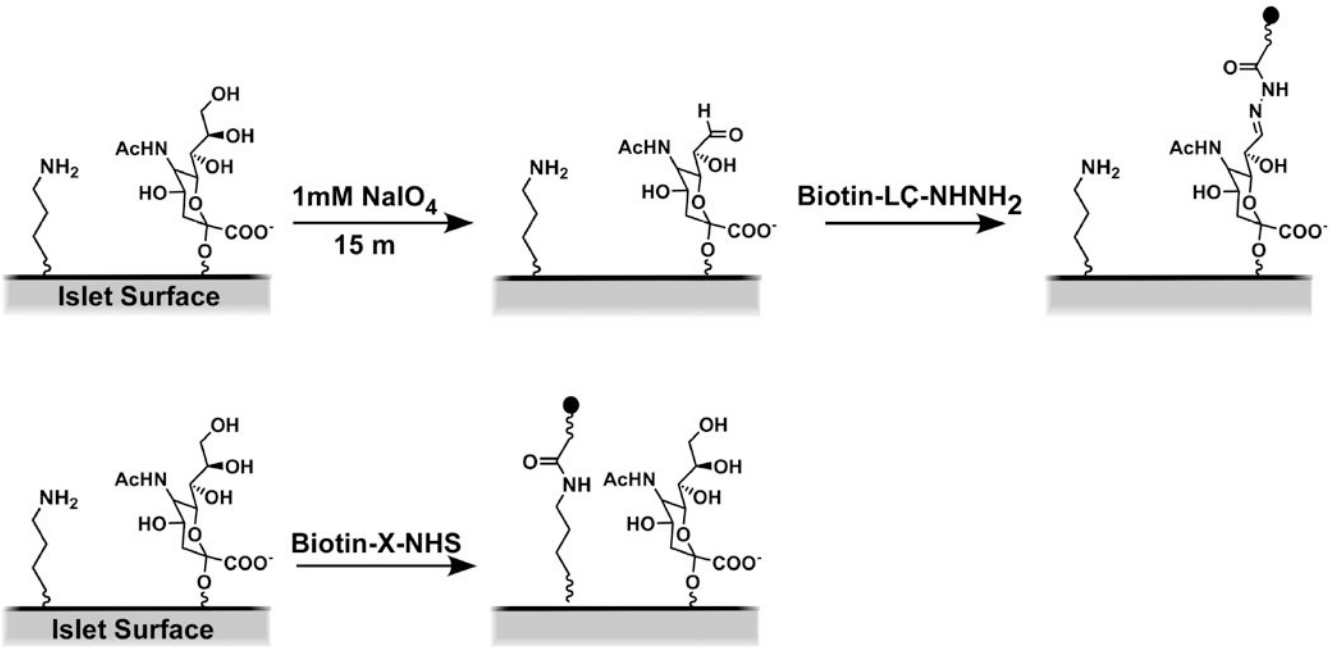


Scheme 1. Re-engineering the islet surface with recombinant thrombomodulin (rTM)
Incorporating rTM on islet surfaces increases their capacity to generate activated protein C (APC).



Scheme 2. Site-specific biotinylation of recombinant human thrombomodulin (rTM) through Staudinger ligation

rTM was engineered with a C-terminal azido group **(1)** and subsequently reacted with triphenylphosphine-$PEG_{3.4kD}$-biotin **(2)**.



Scheme 3. Islet surface biotinylation through chemical targeting of aldehydes and amines

Top: conjugation of biotin (●) via hydrazone bond formation between biotin-hydrazide and aldehydes generated through mild sodium metaperiodate ($NaIO_4$) oxidation of sialic acid residues. **Bottom:** islet biotinylation using NHS-ester functionalized biotinylation reagents.

Table 1

Biotinylation Conditions for Surface Treatment of Murine Islets

Condition	Reagent	Concentration (mM)	Time (hr)
1	NHS-$PEG_{3.4kD}$-biotin	4	1
2	sNHS-LC-biotin	4	1
3	sNHS-LC-biotin	20	1
4	Hydrazide-LC-biotin	4	1
5	Hydrazide-LC-biotin	4	3
6	Hydrazide-LC-biotin	20	1